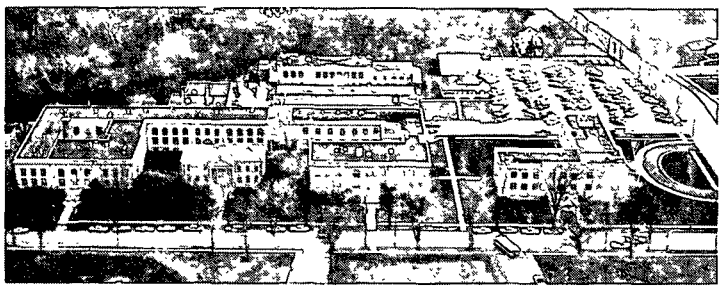

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ENDOGENOUS ANTIOXIDANTS AND ENERGY CONSIDERATIONS IN PINE VS. WILD CARROT SUSPENSION CULTURES

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To assess the basis for the apparent morphogenic incompetence of pine cell suspension cultures, the endogenous concentrations of the water soluble antioxidants, glutathione (GSH) and ascorbic acid (AA), were monitored during the growth of morphogenically unresponsive loblolly pine (Pinus taeda L.) suspension cultures in the presence and absence of 2,4-dichlorophenoxyacetic acid (2,4-D). Relative to wild carrot (Daucus carota L.) cell suspensions which formed somatic embryos in the absence of 2,4-D, the pine cells exhibited markedly different changes in antioxidant content in response to subculture and transfer to 2,4-D-free media. Particularly notable was a transient substantial increase in the antioxidant levels shortly after subculture which is not characteristic of competent wild carrot cells growing with or without 2,4-D. Analogous comparative data on adenosine triphosphate (ATP) content and energy charge suggest that these pine cells may be similar to carrot cells which have lost their developmental potency and show little ATP utilization.

Key words: pine, carrot, suspension cells, antioxidants, energy

Introduction

After many years of relative drought, investigators concerned with plant regeneration from conifer tissue cultures have been reporting some success.

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Abbreviations: GSH, glutathione; AA, ascorbic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; GSSG, oxidized glutathione dimer; DHA, dehydroascorbic acid.

Particularly noteworthy are reports of somatic embryogenesis occurring in Norway spruce [1,2], sugar and loblolly pines [3], and European larch [4]. Despite these advances, attempts to conduct these morphogenic processes in cell suspension cultures, in the mode of now classical wild carrot (Daucus carota L.) somatic embryogenesis [5], appear to remain largely unrewarded.

Since the development of a conifer culture medium in this laboratory a few years ago [6], with routine subculture we have been able to maintain suspension cultures of pine cells for prolonged periods as long as 2,4-D (or some other exogenous auxin) was present. In most cases, the removal of 2,4-D (in mimicry of the protocol for wild carrot somatic embryogenesis) from the medium resulted in cessation of growth and eventual death of the cultures. However, a few cases were encountered, usually with newly initiated cell lines, in which pine cells did continue to grow, albeit proliferatively, upon withdrawal of synthetic auxin from the medium. Cells from both types of cultures, i.e., showing growth or no growth upon transfer to auxin-free medium, were subjected to the biochemical analyses reported here.

The data obtained in this study are interpreted largely in relation to our analogous observations on the wild carrot system, some of which have been reported elsewhere [7,8], which accounts for the choice of the antioxidants, glutathione and ascorbic acid, as subjects for study. Very recently we found that related analyses conducted on embryogenic and nonembryogenic Norway spruce (Picea abies Karst L.) callus (originating from a single explant source) produced data on antioxidants consistent with our findings for the wild carrot cultures [9]. Consequently, the substantial taxonomic gap between wild carrot and conifers has been bridged somewhat, i.e., there is further reason to expect that, were they morphogenically responsive, the pine cells examined here should

show greater similarities to wild carrot cells in the results obtained for these particular parameters.

Considering that the energy status of a cellular system may be strongly linked to its redox state, we have included some data on ATP and energy charge in these pine cells, again in relation to analogous observations on wild carrot cells. As far as we are aware, data of this nature are not available elsewhere and may be of value in dealing with various recalcitrant cultures.

Presented here then are results obtained when morphogenically unresponsive pine cells in suspension were harvested and analyzed for redox and energy parameters used to characterize wild carrot cells which have served as a model of somatic embryogenesis in this laboratory. It was hypothesized that comparison of these data with analogous results for the carrot system would provide insight about possible molecular level malfunctioning in these pine cells.

Materials and methods

Cell suspension cultures

The wild carrot (Daucus carota L.) cells were grown in the dark in Erlenmeyer flasks on gyratory shakers in modified wild carrot medium with subculture, screening, and inoculation for proliferation (with 2,4-D) or somatic embryogenesis (without 2,4-D) as described [7]. Cells or somatic embryos were harvested by filtration and used for fresh weight determinations (in triplicate) prior to analyses for the biochemical parameters. Individual flasks constituted a replicate for the analyses at the various time points rather than aliquoting at sampling times from larger batches.

Loblolly pine (Pinus taeda L.) cell line 10D originated from cotyledons of young seedlings. The callus was initiated during the development of a medium which eventually became known as LM [6]. It was maintained in suspension in the presence of 0.5 mg/L 2,4-D on LM3 which differs from the published LM as follows (in mg/L): 370 rather than 1850 $MgSO_4 \cdot 7H_2O$; 1538 $MgNO_3 \cdot 6H_2O$ (not present in standard LM); 250 rather than 100 myo-inositol. Cell line 2H was initiated also from young seedling cotyledons on LM medium. It was grown in suspension in LM in the presence of 2 mg/L 2,4-D and 0.1 mg/L BA (benzyladenine). Cell line 2F was pooled from calli initiated from several immature zygotic embryos on LM. In the studies reported here it was grown in suspension in LM in the presence of 2 mg/L 2,4-D.

All loblolly pine cell lines were washed and screened (>63 μm) in LM or LM3 (line 10D only) at the time of inoculation. Inoculation was 200 mg per 50 mL of medium in 250 mL Erlenmeyer flasks for these investigations. This is much higher than that for wild carrot cells (0.5 μL packed cells per mL) but necessary to insure the viability of the pine cells in suspension. The pine cell suspensions were grown on a shaker like the carrot cells; however, although cell lines 10D and 2F were grown in the dark like the carrot cells, line 2H was light-grown (23°C, 16 hour irradiance with cool-white fluorescent and incandescent light at 15-50 $\mu Em^{-2}s^{-1}$). The harvesting and replication procedures were essentially those given for wild carrot cells above. All operations with carrot and pine cells up to the point of harvesting and analysis were conducted aseptically.

Analytical procedures

The extraction of the pine and carrot cells and the spectrophotometric assays for GSH and GSSG (oxidized GSH) were adapted from Brehe and Burch as

already described for wild carrot [7]. The same extracts were analyzed for AA and DHA (oxidized AA) by the fluorimetric method of Deutsch and Weeks which was also described elsewhere for wild carrot [8]. ATP, ADP and AMP were all analyzed by the luciferin-luciferase photoluminescence technique [10]. ATP was analyzed directly, whereas ADP and AMP were converted to ATP by commercial enzymes prior to analysis. Cells were harvested periodically as for the other analyses, and the adenylates were extracted with DMSO (dimethylsulfoxide). Energy charge was computed using the formula developed by Atkinson [11].

Triplicates (separate flasks) were used for analyses following the fresh weight determinations as noted above. Data points (constituting individual plotted curves) which are accompanied by the same letter are not significantly different from each other as determined by ANOVA followed by Duncan's new multiple range test ($p < 0.05$).

Results and discussion

At the time that the glutathione data on pine cells was gathered, the growth curves for two of the pine cell lines, both in the presence and absence of added 2,4-D, were as shown in Fig. 1. Note that cell line 2F reached stationary phase by day 13 in the presence of 2,4-D but not in its absence. On the other hand, cell line 2H was just emerging from the lag phase on day 13 in the presence of 2,4-D and failed to grow in its absence.

In the presence of 2,4-D, the GSH content of the two cell lines increased shortly after subculture as shown in Fig. 2. The GSH content of cell line 2F then decreased briefly but had halted its decline at day 10 when this culture had emerged from its lag phase and was growing rapidly. Contrarily, following the initial increase, the GSH content of cell line 2H continued to

fall steadily. It appears likely that a slight recovery in GSH level occurred beyond two weeks when the culture finally began to grow, but the numbers are not statistically significant.

Both cell lines likewise exhibited a transient increase in GSH content, followed by a decrease, after transfer to medium lacking 2,4-D as shown in Fig. 2 also. In this case cell line 2F, which grew in the absence of 2,4-D, increased its GSH content again in concert with the upturn in the growth curve. For cell line 2H, which failed to grow in the absence of 2,4-D, the decline in GSH content continued steadily until GSH reached the verge of disappearance at 23 days. Note that this culture, in the presence of 2,4-D, apparently had not yet reached stationary phase at this time, so taking a data point at day 23 in the absence of 2,4-D was not unreasonable in this case (bolstered by the fact that the point obtained fell into line with a well established straight line function).

GSSG, the oxidized dimeric form of GSH, was also measured in all of the foregoing cases. However, its presence was barely detectable in all cases, so the data are not presented. The maximum amount found was 0.1 μmole/gram fresh weight which occurred at day 16 for cell line 2H in the absence of 2,4-D.

Ascorbic acid (AA), another plentiful water soluble antioxidant, was found to exhibit fluctuations similar to GSH in these cultures. Some of these results were obtained with cell line 2F (above), but the data presented here came from a third cell line, 10D, which was dark-grown and did not grow appreciably upon transfer to 2,4-D-free media (growth curves available but not shown). As can be seen in Fig. 3, like GSH content, AA content also responded to subculture with a transient rise shortly thereafter. Following a decrease, it rebounded somewhat in the second week when the culture began to grow in the

presence of 2,4-D. Note that the reversal at day 8 in the absence of 2,4-D was not statistically significant, although it did plateau and correspond to a very weak upturn in the growth curve. [The analogous two curves (not shown) for cell line 2F, which grew in the presence and absence of 2,4-D, were both very similar to the curve for line 10D in the presence of 2,4-D.]

This cell line (10D) had been maintained in suspension culture for well over two years, and it was analyzed again at a later date when it began to falter and not grow much even in the presence of 2,4-D. As also shown in Fig. 3, at that time AA content still showed a transient though weaker rise immediately after subculture, with no late recovery in concert with little growth. However, perhaps more interesting was an observed transient increase in dehydroascorbic acid (DHA) at the same time. For the most part, this phenomenon occurred independently of the presence of 2,4-D as shown in Fig. 4 where it is compared to data obtained earlier when the cell line was still exhibiting a growth response to 2,4-D.

Prior to the deterioration of cell line 10D just addressed (Fig. 4), it was subjected to analysis for adenine nucleotides (ATP, ADP, AMP). Initially, it was suspected that its failure to grow in the absence of 2,4-D might be related to an energy deficiency. However, it was found (Fig. 5) that, in fact, the energy charge in this culture was extremely high irrespective of the presence of 2,4-D. ATP content <u>per se</u> (Fig. 5) showed a transient increase like the antioxidants, followed by a decline which halted temporarily between days 6 and 10.

It was of value to compare these results with analogous data that we had on hand for the wild carrot system (Fig. 6). Several wild carrot cell lines of varying embryogenic potency have been maintained in this laboratory. Our main wild carrot cell line would occasionally lose its potency (loss of

somatic embryo yield) and require "rejuvenation". Usually this involved starting a callus culture again from somatic embryos. Energy charge and ATP data from wild carrot cells that were morphogenically responsive or unresponsive (Fig. 6) showed differences. Although the unresponsive culture had a relatively low level of ATP at the time of subculture, responsive and unresponsive cultures contained similar quantities of ATP at the end of the first week following subculture. Note, however, that the energy charge values indicate ATP utilization in the responsive culture, whereas in the unresponsive culture they quickly rose to a high value and remained there for the two week duration.

Relative to the findings about fluctuations in GSH and AA contents of embryogenically responsive wild carrot cell suspensions [7,8], these pine cell suspensions exhibited both similarities and differences. The substantial increases in GSH and AA contents seen in pine cell suspensions shortly after subculture are not characteristic of wild carrot cell suspensions. On the other hand, bearing in mind that longer lag phases were often found for pine cell suspensions than for carrot cell suspensions, note that, like carrot, the pine cell suspensions were found to display a peak or plateau in their GSH and AA contents if and when they entered log/linear phase growth but not when they failed to grow. A strong linkage between antioxidants and growth processes is apparent, but more refined studies would be needed to establish the nature of the connection.

Wild carrot cultures usually exhibit peaks of both GSH and AA roughly coincident with log phase growth, and such peaks are smaller in the absence of 2,4-D, a condition leading to the development of somatic embryos [7,8]. Sung [5] has labeled subculture <u>per se</u> as the most important step in the carrot somatic embryogenesis protocol. Regardless of the presence of 2,4-D, the pine cell

response to subculture in terms of endogenous antioxidant levels seems to be ill-timed when viewed in juxtaposition with carrot cells that are morphogenically competent. The immediate increase in antioxidant concentrations in the pine cells may be indicative of an attempt to counter shock and stress. Even this response seemed to fail in the weakened cell line as it produced DHA (Fig. 4). Elsewhere [8] we have presented data which indicate that elevation of cellular antioxidant levels may be part of the mode of action of 2,4-D in the wild carrot tissue culture system. This line of reasoning relative to 2,4-D action was pursued in pretissue culture times by Key and coworkers, but it was finally abandoned in 1967 [12].

The observed changes in GSH and AA concentrations in the pine cells following subculture occurred in a concentration range about tenfold lower than those observed in wild carrot cells. Whether this was a function of the *in vitro* culture conditions for the pine cells versus the carrot cells or a species difference is not known. However, Esterbauer and coworkers [13,14] have reported GSH and AA values for spruce needles (on a fresh weight basis) that were lower than found here in the case of GSH, and higher than found here in the case of AA. Our own analyses of these parameters in developing *Pinus resinosa* Ait. and *Pinus strobus* L. ovules revealed somewhat lower levels for both GSH and AA [15]. Therefore, our attention has focused not so much on the absolute quantities of these antioxidants as on the relative amounts present at different stages of culture.

The strong similarity between morphogenically unresponsive wild carrot cells and these pine cells with respect to the energy charge (Fig. 6 *vs.* Fig. 5) may warrant further investigation. The only apparent difference between these two results is that the unresponsive carrot culture took a little longer time (still less than one day) to attain the extremely high energy charge value. The

sheer presence of chemical energy in the form of ATP would not appear to be a problem for either of these systems; making use of it might be.

All of the data on antioxidants and energy parameters presented here are expressed on a fresh weight basis. We have considered the possibility that some other basis might color interpretation of the observed phenomena. However, using a Coulter counter, we have determined cell numbers for both pine and carrot cells under these conditions. Although the pine cells are larger than carrot cells, and, therefore, there are fewer of them per unit mass, we found that plots of cells/mg fresh weight vs. time yield virtually horizontal straight lines over a culture period for both species. Consequently, we are reasonably confident that at least conversion to a per cell basis would have little impact on the data presented here.

It is noteworthy that slime mold development has been linked to changes in GSH content in a manner very similar to wild carrot [16]. Results of our studies on pine zygotic embryo development [15] would appear to be more in line with the carrot system than with these pine cell suspensions. Since our recent findings on Norway spruce embryogenic vs. nonembryogenic callus [9] also suggest that Norway spruce resembles wild carrot in terms of the antioxidant parameters measured here, it is reasonable to expect that an embryogenic suspension culture of pine cells would show greater similarities to embryogenic wild carrot cells than were observed in the current investigation. Now that embryogenic conifer callus is available, we are optimistic that analogous data for embryogenic pine and spruce suspension cells can be obtained which may reinforce the apparent commonality of this behavior.

It is concluded that these pine cell suspensions lack a degree of redox control characteristic of wild carrot cell suspensions capable of somatic

embryogenesis. Furthermore, there are indications that ATP utilization is impaired in these cultures. It will be of considerable interest if conifer cell suspensions can be obtained that act more like embryogenic carrot cells with respect to these parameters.

Acknowledgments

The authors would like to thank the personnel of the Tissue Culture Laboratory at The Institute of Paper Chemistry for their assistance in tissue culture aspects of this investigation.

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FIGURE LEGENDS

Figure 1. Growth curves for loblolly pine cell lines 2F (circles) and 2H (triangles) in the presence (○ or △) and absence (● or △) of 2,4-D. All data points are triplicate means except those at day 23 for cell line 2F which were unreplicated and are connected by dotted lines. ANOVA followed by Duncan's new multiple range test was used to compare the means within each curve for significance ($p < 0.05$); data points within a single curve are not significantly different from one another if they have common letters.

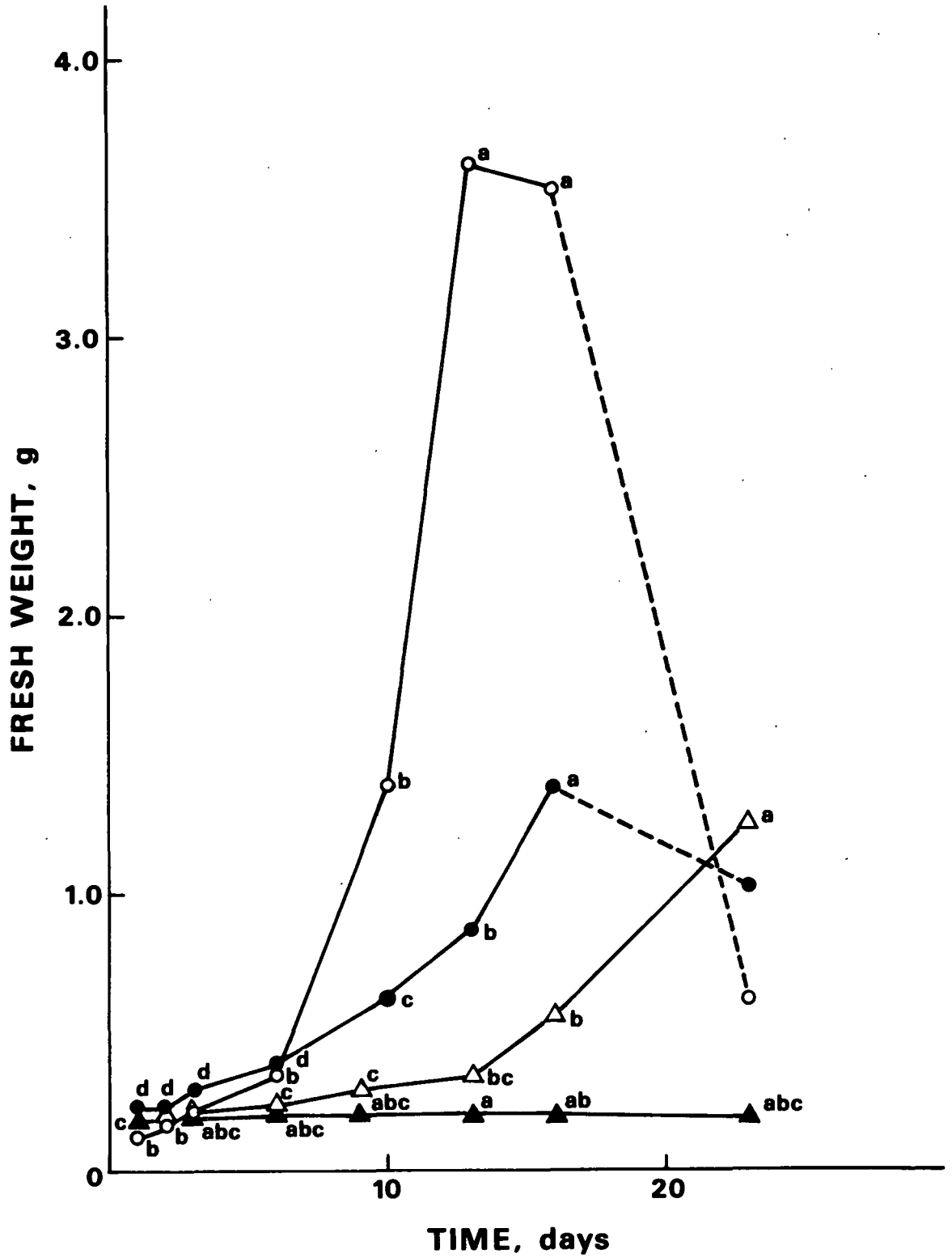
Figure 2. GSH content of loblolly pine cell lines 2F and 2H as a function of time from subculture in the presence and absence of 2,4-D. Symbols and statistical analysis as in Figure 1.

Figure 3. AA content of loblolly pine cell line 10D when normal (solid line) and deteriorating (dotted line) as a function of time from subculture in the presence (○) and absence (●) of 2,4-D . Statistical analysis as in Figure 1.

Figure 4. DHA content of loblolly pine cell line 10D when normal (solid line) and deteriorating (dotted line) as a function of time from subculture in the presence (○) and absence (●) of 2,4-D. Statistical analysis as in Figure 1.

Figure 5. Energy charge (top) and ATP content (bottom) of loblolly pine cell line 10D as a function of time from subculture in the presence (○) and absence (●) of 2,4-D. Statistical analysis as in Figure 1.

Figure 6. Energy charge (top) and ATP content (bottom) of wild carrot cells as a function of time from subculture in the presence (○) and absence (●) of 2,4-D; responsive culture (left) and unresponsive culture (right). Statistical analysis as in Figure 1.



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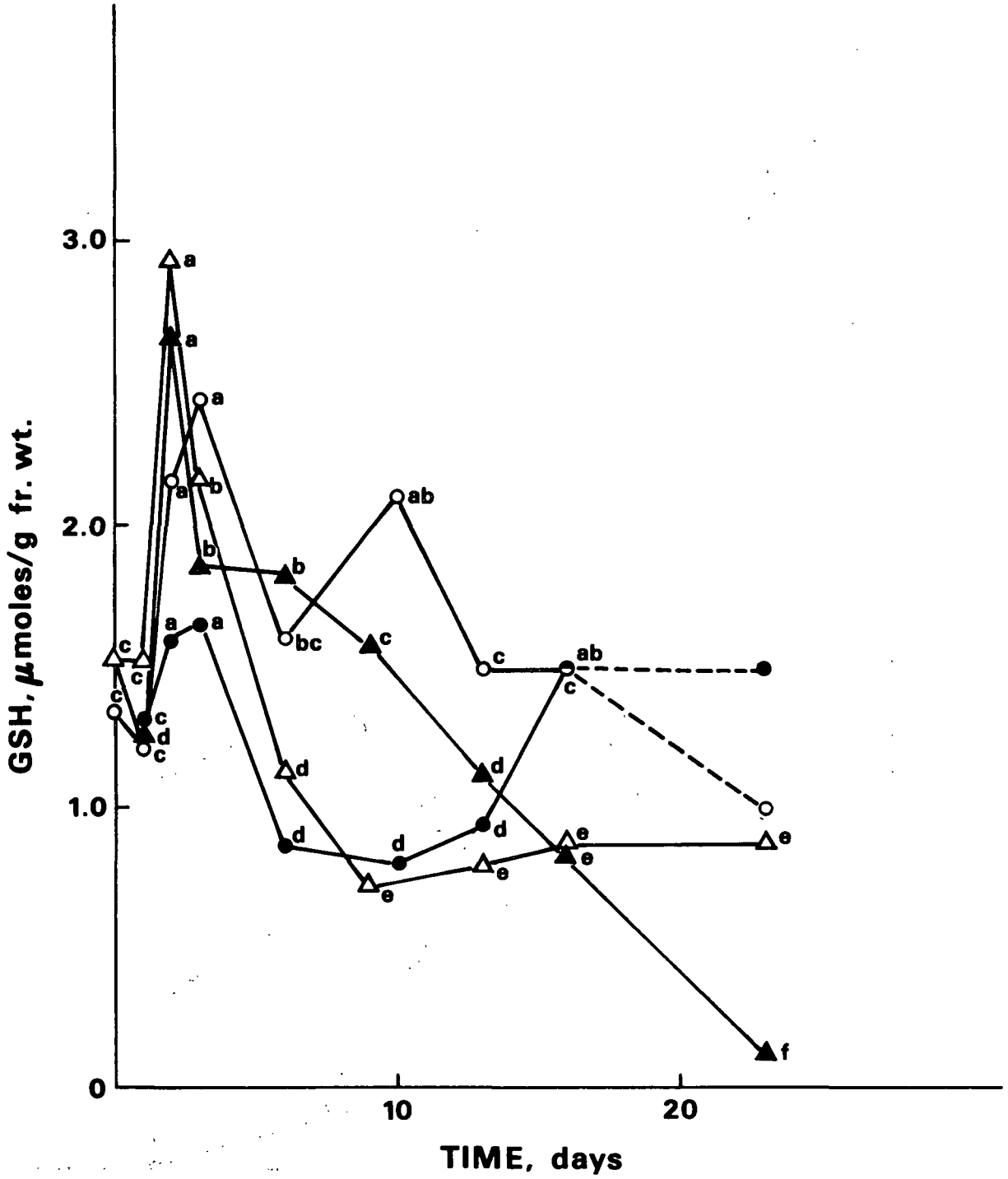

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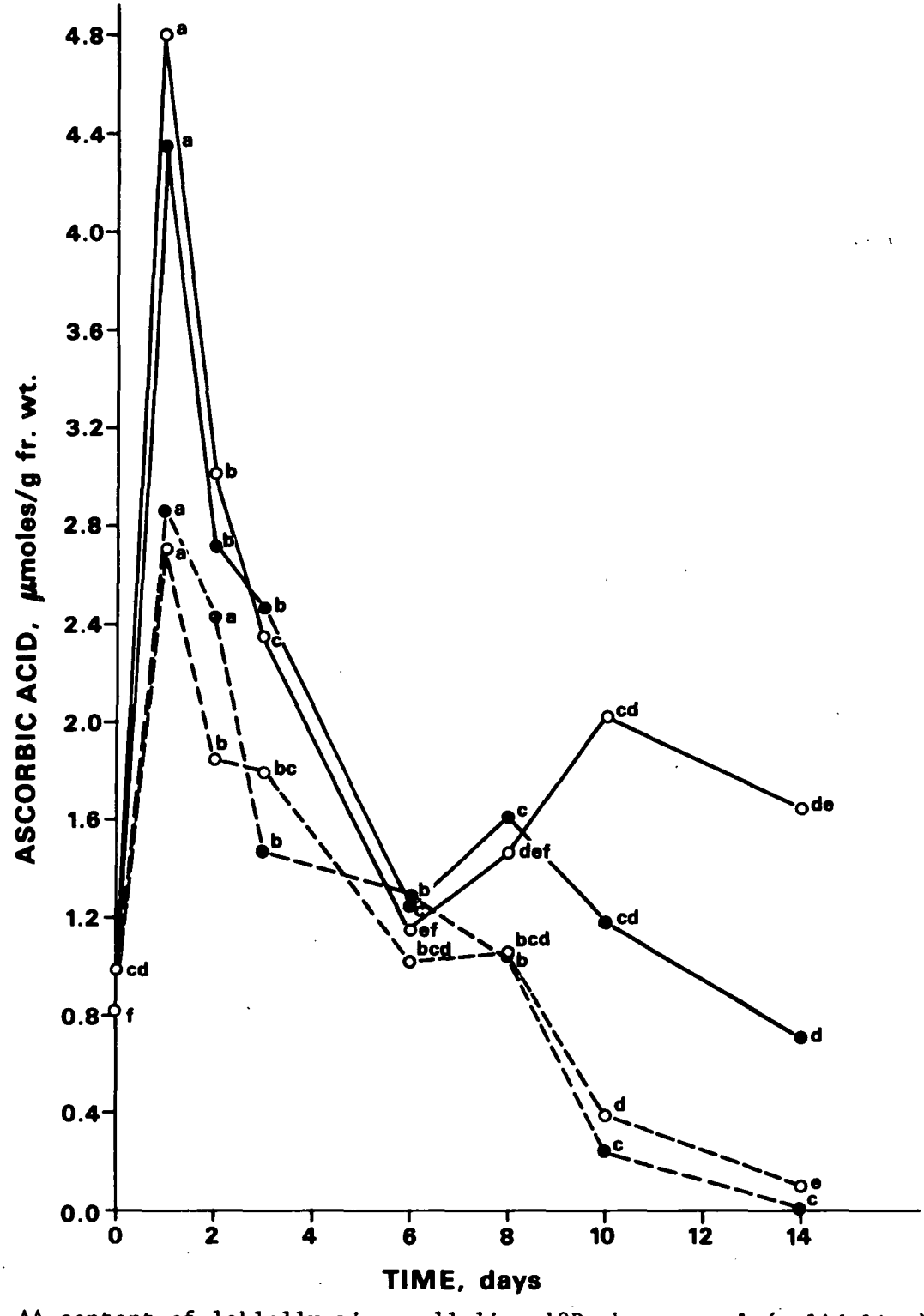

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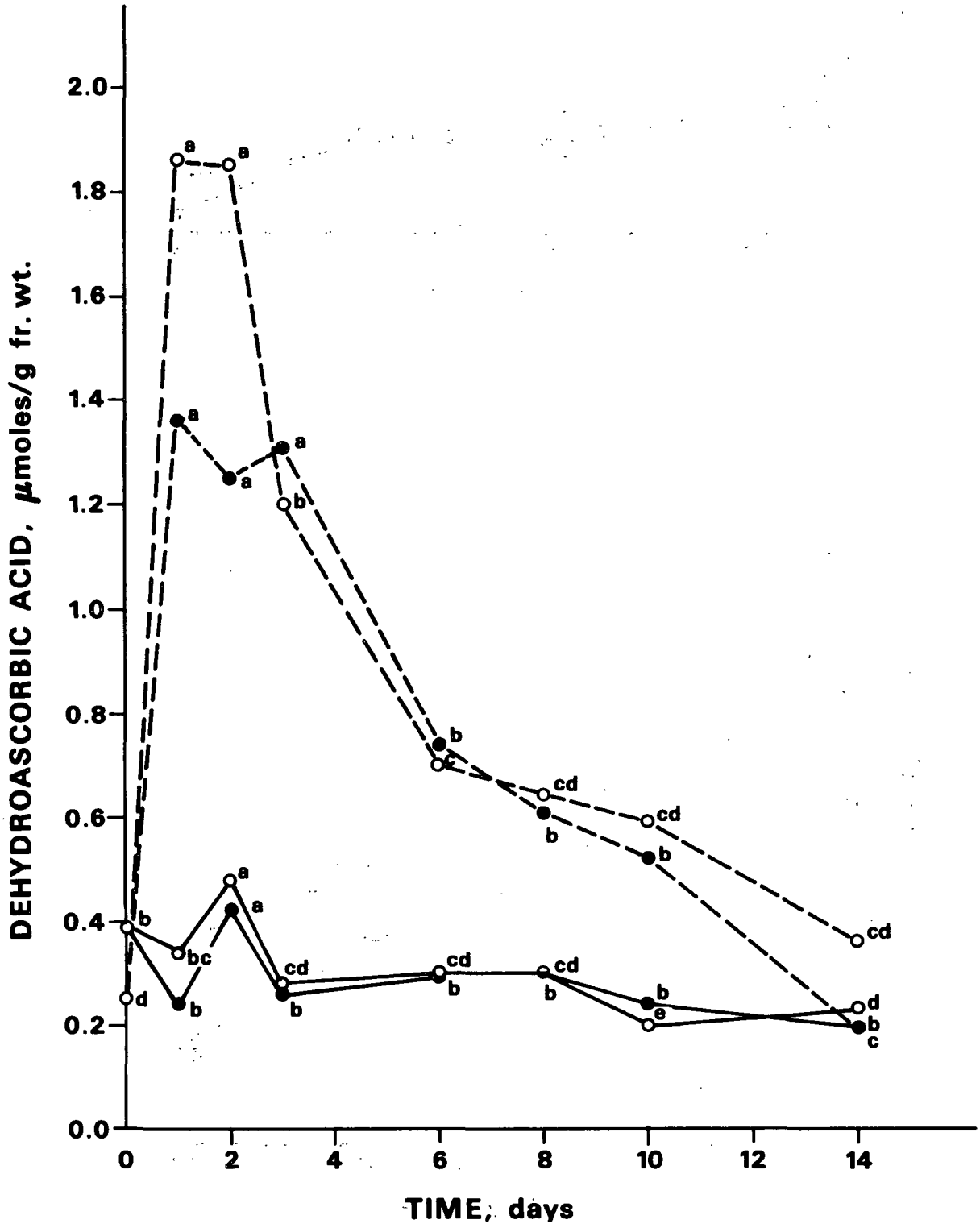

Figure 4. DHA content of loblolly pine cell line 10D when normal (solid line) and deteriorating (dotted line) as a function of time from subculture in the presence (○) and absence (●) of 2,4-D. Statistical analysis as in Figure 1.

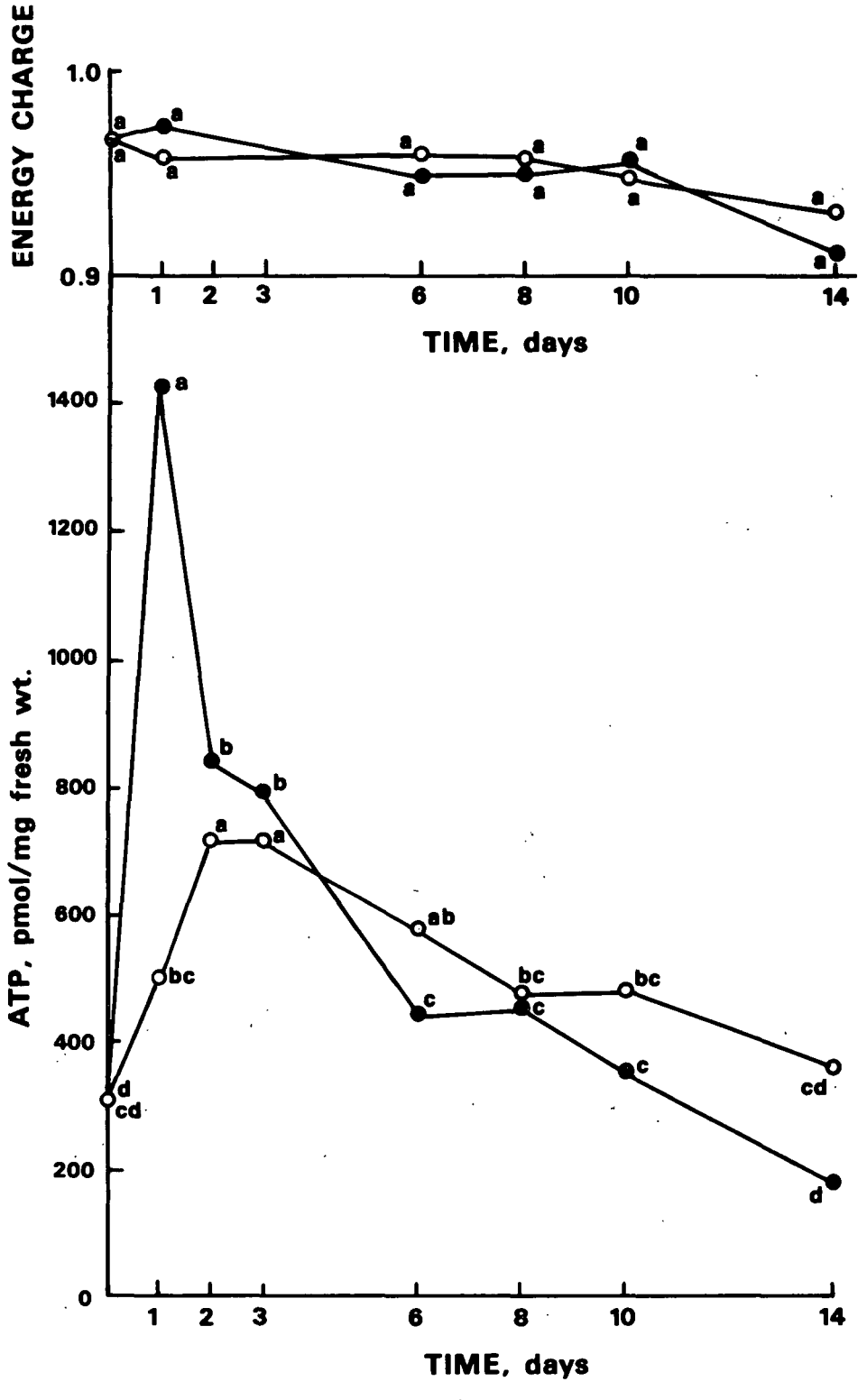

Figure 5. Energy charge (top) and ATP content (bottom) of loblolly pine cell line 10D as a function of time from subculture in the presence ( ) and absence (●) of 2,4-D. Statistical analysis as in Figure 1.

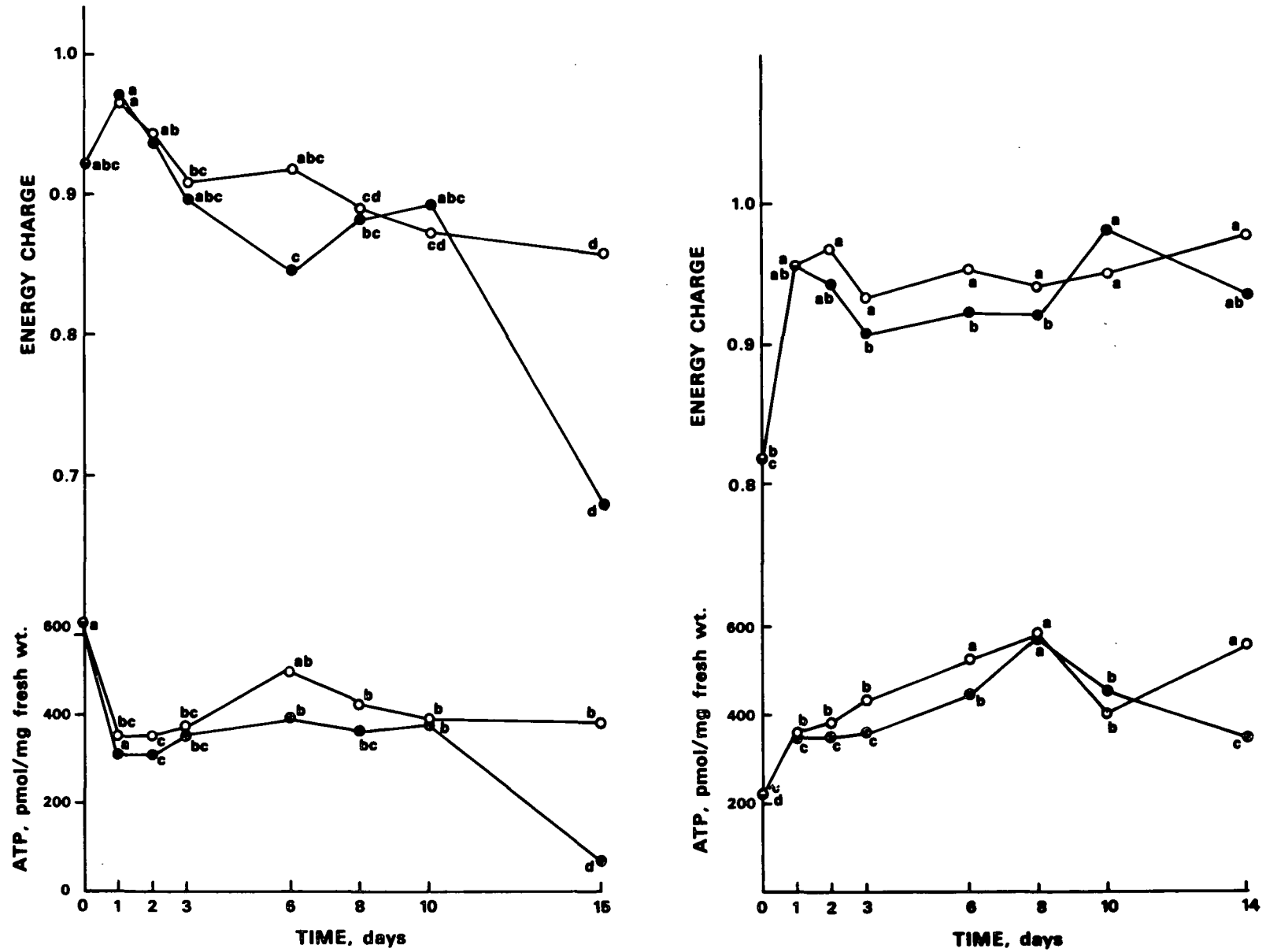

Figure 6. Energy charge (top) and ATP content (bottom) of wild carrot cells as a function of time from subculture in the presence ( ) and absence (●) of 2,4-D; responsive culture (left) and unresponsive culture (right). Statistical analysis as in Figure 1.